

# Activation of Protein Kinases in Chronically Hypoxic Infant Human and Rabbit Hearts Role in Cardioprotection

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**Background**—Many infants who undergo heart surgery have a congenital cyanotic defect in which the heart is chronically perfused with hypoxic blood. However, the signaling pathways by which infant hearts adapt to chronic hypoxia and resist subsequent surgical ischemia is unknown.

**Method and Results**—We determined the activation and translocation of protein kinase C (PKC) isoforms and mitogen activated protein kinases (MAP kinases) in 15 infants with cyanotic ( $Sao_2 < 85\%$ ) or acyanotic ( $Sao_2 > 95\%$ ) heart defects undergoing surgical repair and in 80 rabbits raised from birth in a hypoxic ( $Sao_2 < 85\%$ ) or normoxic ( $Sao_2 > 95\%$ ) environment. Tissues from infant human and rabbit hearts were processed for Western and *in vitro* kinase analysis. In human infants with cyanotic heart defects, PKC $\epsilon$ , p38 MAP kinase, and JUN kinase but not p42/44 MAP kinase were activated and translocated from the cytosolic to the particulate fraction compared with acyanotic heart defects. In rabbit hearts there was a parallel response for PKC $\epsilon$ , p38 MAP kinase, and JUN kinase similar to humans. In infant rabbit hearts inhibition of PKC $\epsilon$  with chelerythrine, p38 MAP kinase, with SB203580 and JUN kinase with curcumin abolished the cardioprotective effects of chronic hypoxia but had no effects on normoxic hearts.

**Conclusions**—Infant human and rabbit hearts adapt to chronic hypoxia through activation of PKC $\epsilon$ , p38 MAP kinase, and JUN kinase signal transduction pathways. These pathways may be responsible for cardioprotection in the chronically hypoxic infant rabbit heart. (*Circulation*. 2002;106:239-245.)

**Key Words:** hypoxia ■ ischemia ■ proteins ■ heart defects, congenital ■ heart diseases

Many infants who undergo cardiac surgery have a congenital cyanotic defect in which the heart is chronically perfused with hypoxic blood. However, the signaling pathways by which infant hearts adapt to chronic hypoxia and resist subsequent surgical ischemia is unknown.

By elucidating the impact that prolonged periods of hypoxia exerted on resistance to subsequent ischemia, we should be able to improve cardioprotection in infants with congenital heart defects.

Protein kinase C (PKC) family members are important mediators of hypoxia. In cardiomyocytes, PKC $\alpha$  and PKC $\epsilon$  translocate from soluble to particulate fractions of the cell in response to the stress of chronic hypoxia.<sup>1</sup> The mitogen-activated protein kinases (MAP kinases) are ubiquitous proteins activated by diverse stimuli and appear to mediate cellular responses including proliferation, differentiation, and adaptation to stress.<sup>2</sup> Three major MAP kinase families have been characterized, including the extracellular signal-regulated kinases (ERK or p42/44 MAPK), the c-Jun NH<sub>2</sub>-terminal

kinases (JUN kinase), and the p38 MAP kinases (p38 MAPKs).<sup>2</sup> ERKs are mainly involved in mediating anabolic processes such as cell division, growth, and differentiation; the JUN kinases and the p38 MAPK are generally associated with cellular response to diverse stresses. The clinical relevance of protein kinases in adult humans was recently demonstrated by an increased activity of JUN kinase and p38 MAPK in heart failure secondary to ischemic heart disease<sup>3</sup> and during cardiopulmonary bypass.<sup>4</sup> However, the role of PKC and MAPKs in the mechanisms by which infant hearts adapt to chronic hypoxia and resist subsequent surgical ischemia are unknown.

To examine the role of these signaling pathways in adaptation to chronic hypoxia we identified and characterized PKC and MAPKs in hearts from human infants with cyanotic ( $Sao_2 < 85\%$ ) or acyanotic ( $Sao_2 > 95\%$ ) heart defects and in hearts from infant rabbits raised from birth in a hypoxic ( $Sao_2 < 85\%$ ) or normoxic ( $Sao_2 > 95\%$ ) environment. We then determined the contribution of PKC and MAPKs to

Received March 21, 2002; revision received April 26, 2002; accepted May 1, 2002.

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*Circulation* is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000022018.68965.6D

**Patient Characteristics**

	Cyanotic (n=7)	Acyanotic (n=8)
Age, mo		
Mean	4.8±0.9	5.8±1.2
Range	1 wk to 9 mo	1 to 10 mo
Body weight, kg	3.9±0.4	5.2±0.5*
Sex, male/female	4/3	4/4
Pathology		
CAVC	0	2
VSD	0	2
TOF	2	0
AS	0	1
DORV	1	0
PAVC	0	3
HLHS	4	0
Hemoglobin, g/dL	15.6±0.6	12.5±1.3*
Blood O <sub>2</sub> saturation, %	73±5	98±1*

CAVC indicates complete atrioventricular canal; VSD, ventricular septal defect; TOF, tetralogy of Fallot; AAVC, atrioventricular septal defect; AS, aortic stenosis; DORV, double-outlet right ventricle with transposition of the great arteries; ASD, atrial septal defect; PAVC, partial atrioventricular canal; and HLHS, hypoplastic left heart syndrome.

\*P<0.05, cyanotic vs acyanotic.

cardioprotection in chronically hypoxic and normoxic infant rabbit hearts. Our studies reveal that many of the protein kinase signaling mechanisms activated by chronic hypoxia in infant rabbits are identical to those activated by cyanotic heart defects in human infants. Once activated, we show that protein kinases confer cardioprotection in the chronically hypoxic infant rabbit heart.

**Methods****Humans**

The use of human tissue in this study was approved by the Human Research and Review Committee at Children's Hospital of Wisconsin and the Medical College of Wisconsin. Fifteen infants undergoing elective open heart surgery for congenital heart defects were prospectively recruited for this study. To determine whether protein kinases are activated by chronic hypoxia, the patients were divided into cyanotic and acyanotic groups according to blood oxygen saturation (cyanotic, SaO<sub>2</sub>>95%; acyanotic, SaO<sub>2</sub><85%). All acyanotic patients were stable, with SaO<sub>2</sub><85% for 24 hours before surgery. There were no emergency operations performed on acutely hypoxic patients. Right atrial tissue (>200 mg) from infants with congenital acyanotic and cyanotic heart defects was harvested at the time of surgical repair. The tissue was immediately frozen in liquid nitrogen and processed for Western analysis as described previously.<sup>3</sup> Preoperative characteristics are summarized in the Table.

**Rabbits**

Animals used in this study received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" formulated by the National Research Council, 1996. Infant rabbits were maintained for 10 days in a hypoxic (SaO<sub>2</sub><85%) or normoxic (SaO<sub>2</sub>>95%) environment as described previously.<sup>6</sup>

**Isolated Heart Perfusion**

Isolated rabbit hearts (n=8/group) perfused in a retrograde manner and instrumented as previously described.<sup>6</sup>

**Effect of PKC and MAPK Inhibitors**

Hearts from normoxic or chronically hypoxic rabbits were perfused in the Langendorff mode. Biventricular function and coronary flow were recorded under steady-state conditions.<sup>6</sup> Hearts were then perfused for 15 minutes with vehicle, chelerythrine (1 μmol/L), SB203580 (15 μmol/L), curcumin (10 μmol/L), or PD98059 (10 μmol/L) before 30 minutes of global normothermic (39°C) ischemia and 40 minutes of reperfusion. Recovery of developed pressure was expressed as a percentage of its predrug, preischemic value. Results are expressed as mean±SD.

To determine the effect of chelerythrine and SB203580 on protein kinases in chronically hypoxic and normoxic hearts, isolated hearts (n=4 to 7 per group) were aerobically perfused with these drugs for 15 minutes. The free wall of the left ventricle was then processed to obtain cytosolic and particulate fractions<sup>7</sup> for Western analysis, as described previously.<sup>5</sup>

**SDS-PAGE and Western Blot Analysis**

Equal concentrations of protein were analyzed by SDS-PAGE and Western blotting by using either isoform-specific antibodies for phospho-PKC detection or specific antibodies against phosphorylated and nonphosphorylated p38 MAPK, JNK, and p42/44 MAPK (Cell Signaling Technology). The blots were developed by ECL. Densitometry was performed on each sample and analyzed with the use of NIH image software. Phosphorylated Hsp27 and PKCε were detected with the use of specific antibodies from Upstate Biotechnology Inc. Total PKC activity was measured by a PKC kit from Amersham, according to the manufacturer's instructions.

**Immunoprecipitation and In Vitro Kinase Assays**

To determine MAPK activity, nonradioactive kinase assay kits were used (Cell Signaling). p38 MAPK activation in normoxic and hypoxic infant human hearts was determined by measurement of its catalytic activity with the use of the in-gel kinase assay using GST-MAPKAPK-2, Hsp27, and GST-ATF-2 as substrate according to the manufacturer's instructions.

**Phosphorylation of Threonine 71 on ATF-2**

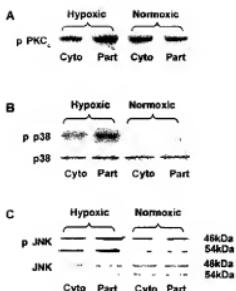
Aliquots of nuclear and cytosolic fractions were subjected to Western analysis with the use of specific phospho-ATF-2 (Thr71) antibody or control anti-ATF-2 as described previously.<sup>8</sup> The purity of the fractions was confirmed with antibody markers specific for the cytosolic and nuclear compartments β-actin and histone deacetylase-1, respectively, with separation confirmed by Western analysis.<sup>9</sup>

**Statistical Analysis**

Statistical analysis was performed by use of repeated measures ANOVA with the Greenhouse-Geisser adjustment used to correct for the inflated risk of a type I error.<sup>5</sup> If significant, the Mann-Whitney test was used as a second step to identify which groups were significantly different. After ANOVA the data were analyzed for differences related to multiple comparisons.<sup>6</sup> Significance was set at P<0.05.

**Results****Adaptation to Chronic Hypoxia****PKC and MAPK in Human Heart**

To determine the involvement of PKC and MAPKs in normoxic and hypoxic hearts, cytosolic and particulate fractions were examined by SDS-PAGE and Western analysis with the use of specific monoclonal and polyclonal antibodies. Our results indicate that in normoxic hearts, multiple PKC isoforms (α, β, γ, ε, δ, and ζ) are present in the cytosolic fractions. However, adaptation to chronic hypoxia results only in the translocation of PKCε from cytosolic fraction to the particulate fraction (Figure 1).



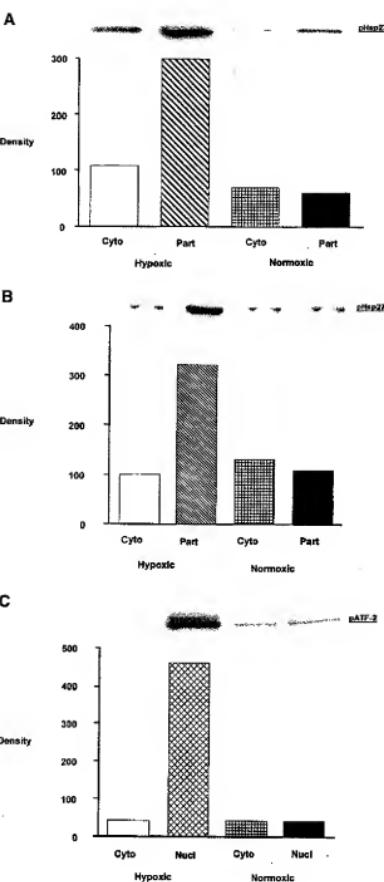
**Figure 1.** Chronic hypoxia in infant human heart results in phosphorylation and translocation of PKC $\epsilon$ , p38 MAP kinase, and JNK kinase from cytosolic to particulate fraction. Cytosolic and particulate fractions were analyzed by Western blotting using phospho-specific antibodies against A, PKC $\epsilon$ ; B, p38 MAPK; and C, JNK. Nonphosphorylated antibodies were used to confirm equal loading of proteins for p38 MAPK and JNK. Cyto indicates cytosolic; Part, particulate.

Next, we sought to determine if the MAPK pathways play a role in adaptation to chronic hypoxia. We have shown that in normoxic hearts, phospho-p38 MAPK is present in both cytosolic and particulate fractions, but chronic hypoxia results in an increase of phospho-p38 MAPK in the particulate fraction (Figure 1). We also found that chronic hypoxia activates JNK kinase in human heart. Chronic hypoxia did not result in activation of phospho-p42/44 MAPK in human hearts. We confirmed that equal amounts of p38 and JNK proteins were analyzed by stripping and reprobing the same blots with control anti-p38 and anti-JNK antibodies (Figure 1).

We examined whether activation and translocation of PKC $\epsilon$ , p38 MAPK, and JNK kinase was related to the variability in clinical presentation of the two groups of patients studied (Table). In all hearts adapted to chronic hypoxia, there was activation and translocation of protein kinases. In contrast, activation and translocation did not occur in any of the normoxic hearts. Thus, in all cases, the changes we observed in protein kinase activation and translocation were solely dependent on oxygen deprivation and not to the underlying clinical presentation responsible for the congenital defect.

p38 MAPK plays a protective role during adaptation to ischemic preconditioning by phosphorylating MAPKAPK-2, which in turn phosphorylates Hsp27.<sup>10</sup> Activation of this pathway is cardioprotective and overexpression of Hsp27 confers protection against ischemia in myocytes.<sup>11</sup> To determine if this pathway is present in human infants and activated by adaptation to chronic hypoxia, we probed normoxic and hypoxic hearts for changes in MAPKAPK-2 and Hsp27. Chronic hypoxia induced activation and translocation of both MAPKAPK-2 and Hsp27 from the cytosolic to the particulate fraction. Neither MAPKAPK-2 nor Hsp27 was activated in normoxic hearts (Figure 2).

p38 MAPK also transduces signals from the cytoplasm to the nucleus in response to cellular stress. ATF2 is a transcrip-



**Figure 2.** Chronic hypoxia in infant human heart activates MAPKAPK-2 and phosphorylates Hsp27 and ATF-2 (Thr 71). A, *In vitro* kinase assay shows phosphorylation of substrate Hsp27 by MAPKAPK-2 in particulate fraction. B, Chronic hypoxia-induced Hsp27 phosphorylation in particulate fraction. C, Chronic hypoxia results in ATF-2 (Thr 71) phosphorylation in nuclear fraction. Cyto indicates cytosolic; Part, particulate; and Nucl, nuclear.

tion factor phosphorylated by p38 MAPK.<sup>8</sup> To determine if this holds in hearts adapted to chronic hypoxia, phosphorylation of GST-ATF2 by p38 MAPK was determined in hearts from normoxic and chronically hypoxic infants. Our results demonstrate that phospho-p38 MAPK immunoprecipitates

from chronically hypoxic hearts result in phosphorylation of GST-ATF-2 in the particulate fraction (Figure 2).

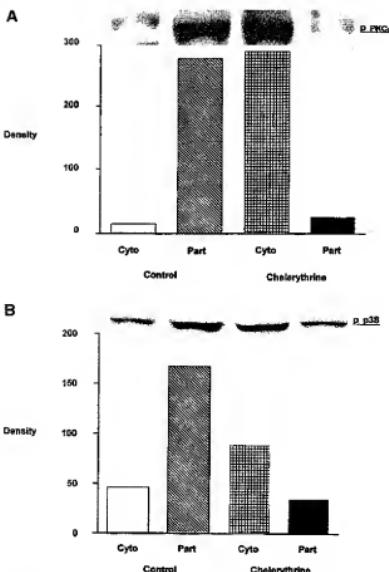
#### ATF-2 Phosphorylation in Nuclear Fraction of Hypoxic Hearts

Transcriptional activity of ATF-2 can be stimulated by JNK and p38 MAPK. ATF-2 binds to both AP-1 and cAMP response element. Therefore, we examined whether chronic hypoxia phosphorylates and activates ATF-2. Our results show that adaptation to chronic hypoxia phosphorylates Thr71 of ATF-2 in the nuclear fraction (Figure 2), suggesting activation of this transcription factor. We confirmed that equal amounts of ATF-2 protein were analyzed by stripping and reprobing the same blots with control anti-ATF-2 antibody.

#### PKC and MAPK in Rabbit Heart

We found an identical pattern of activation for PKC $\epsilon$ , and MAPKs in isolated perfused hearts from rabbits adapted to chronic hypoxia. Chronic hypoxia also induced activation of both MAPKAP-2 and Hsp27 in the particulate fraction. This pattern of activation was also present in freshly excised hearts not subjected to perfusion before analysis. To determine the relative upstream/downstream positions of PKC $\epsilon$ , p38 MAPK, and JUN kinase in the signal transduction pathway activated by chronic hypoxia, hearts were perfused with specific inhibitors of PKC and p38 MAPK, followed by Western blot analysis of the heart lysates. Perfusion of isolated rabbit hearts with chelerythrine, an inhibitor of PKC, reversed the translocation of PKC $\epsilon$ , p38 MAPK, and JUN kinase in chronically hypoxic rabbits but had no effect in normoxic rabbits (Figure 3). Perfusion of hearts with SB203580, an inhibitor of p38 MAPK, also reverses the translocation of p38 MAPK but not PKC $\epsilon$  or JUN kinase in chronically hypoxic hearts. SB203580 had no effect in normoxic rabbit hearts (Figure 4). These data suggest PKC $\epsilon$  is an upstream kinase for activation of p38 MAPK and JUN kinase in chronically hypoxic rabbit hearts. SB20380 also prevented activation of ATF-2 by p38 MAPK in chronically hypoxic hearts. Our data shows that many of the protein kinase signaling mechanisms activated by chronic hypoxia in infant rabbit hearts are identical to those activated by cyanotic heart defects in infant human hearts.

We determined whether protein kinase activation in chronically hypoxic rabbit hearts is altered by subsequent perfusion with bicarbonate buffer. Excised hearts not subjected to subsequent perfusion and excised hearts subjected to 45 minutes of aerobic perfusion were freeze-clamped. Western analysis of PKC $\epsilon$  and p38 MAPK revealed no differences in the extent of activation between the two groups. These data indicate the initial period of perfusion exerted no effect on protein kinase activation. To determine the ability of curcumin to specifically inhibit JNK rather than p38 MAPK normoxic hearts were perfused with anisomycin (20  $\mu$ mol/L). Curcumin (10  $\mu$ mol/L) completely blocked anisomycin-induced phosphorylation of JNK and minimally blocked phosphorylation of p38 MAPK. These data indicate curcumin selectively inhibits JNK with minimal effects on p38 MAPK (Figure 5).



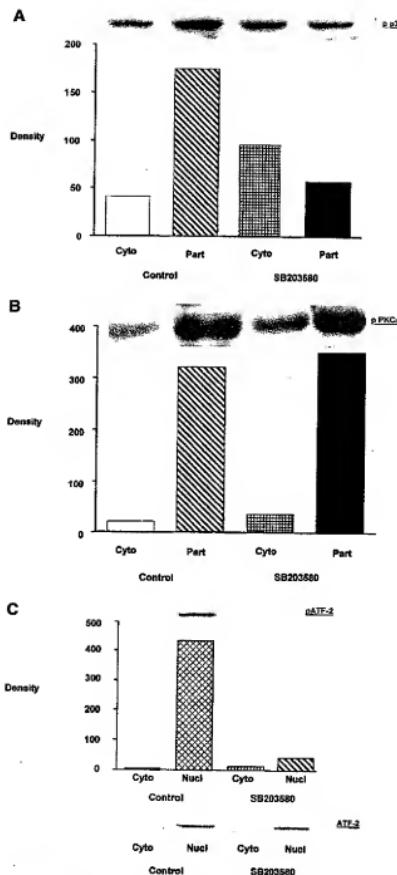
**Figure 3.** Effect of chelerythrine, a PKC inhibitor, on PKC $\epsilon$  and p38 MAPK in chronically hypoxic rabbit heart. Cytosolic and particulate fractions were analyzed by Western blotting with specific antibodies against A, phospho-PKC $\epsilon$ , and B, phospho-p38 MAPK. Chelerythrine significantly inhibited translocation of both PKC $\epsilon$  and p38 MAPK from cytosolic to particulate fraction in hypoxic rabbit heart. Cyto indicates cytosolic; Part, particulate.

#### Parallel Response to Right Atria and Left Ventricle to Chronic Hypoxia

Human atrial but not ventricular tissues were readily obtainable for study. In contrast, rabbit ventricular and atrial tissue were both readily obtainable. However, we did not know if the adaptive response of left ventricle to chronic hypoxia parallels that of right atria. The degree of chronic hypoxia in the atria may not reflect that of the ventricle. We determined the impact of chronic hypoxia on PKC $\epsilon$  and p38 MAPK activation and translocation in left ventricle and right atria from chronically hypoxic rabbits. Chronic hypoxia resulted in activation and translocation of PKC $\epsilon$  and p38 MAPK in both left ventricle and right atria (Figure 6). These data demonstrate right atrial tissue responded to the same extent as left ventricle to chronic hypoxia. Thus, right atria are suitable to study chronic hypoxia-induced changes in protein kinase activation.

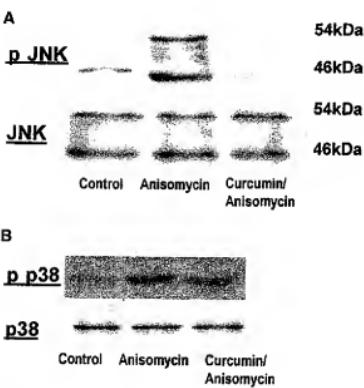
#### Resistance to Ischemia

Cardiac function and the effects of protein kinase inhibitors on aerobic function before ischemia were determined in infant normoxic and chronically hypoxic rabbit hearts. Coronary flow rate was 18% higher in hypoxic hearts than



**Figure 4.** Effect of SB203580, a p38 MAPK inhibitor, on p38 MAPK, PKC $\epsilon$ , and ATF-2 in chronically hypoxic rabbit heart. Cytosolic, particulate, and nuclear fractions were probed with specific antibodies against A, phospho-p38 MAPK; B, phospho-PKC $\epsilon$ ; and C, phosphorylated and nonphosphorylated ATF-2. SB203580 inhibits translocation of p38 MAPK from cytosolic to particulate fraction in hypoxic rabbit heart but did not inhibit translocation of PKC $\epsilon$ . SB203580 inhibits phosphorylated but not nonphosphorylated ATF-2 in the nuclear fraction of hypoxic rabbit heart. Cyto indicates cytosolic; Part, particulate; and Nucl, nuclear.

normoxic controls as an adaptive response to increased oxygen delivery to the myocardium. Right ventricular developed pressure was higher in chronically hypoxic hearts than in normoxic hearts as a consequence of right ventricular

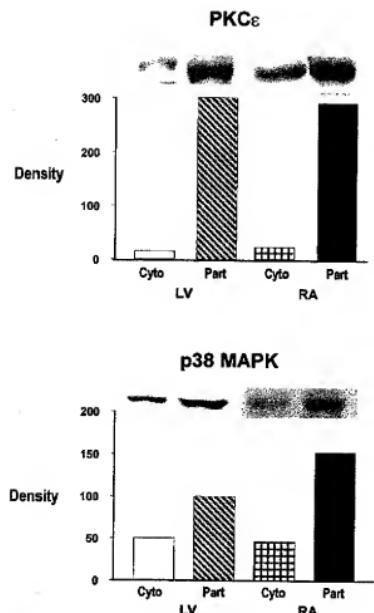


**Figure 5.** Effect of curcumin on JNK kinase and p38 MAPK in normoxic rabbit heart. Isolated hearts were perfused with anisomycin alone (20  $\mu$ mol/L) for 15 minutes and then with anisomycin (20  $\mu$ mol/L) plus curcumin (10  $\mu$ mol/L) for 15 minutes. Cell lysates were probed with specific antibodies against A, JNK, and B, p38 MAPK. Anisomycin activated JNK and p38 MAPK. Curcumin completely blocked phospho-JNK activation and minimally blocked phospho-p38 MAPK activation. P indicates phosphorylated antibody.

hypertrophy. Chelerythrine (1  $\mu$ mol/L), SB203580 (15  $\mu$ mol/L), curcumin (10  $\mu$ mol/L), and PD98059 (10  $\mu$ mol/L) did not exert any effect on heart rate, coronary flow, or developed pressure in left or right ventricle in normoxic or chronically hypoxic hearts before ischemia. To determine the effect of chronic hypoxia on resistance to myocardial ischemia, recovery of postischemic function, was examined in infant normoxic and hypoxic hearts not subjected to drug intervention. Recovery of developed pressure in the left ventricle after ischemia was greater in chronically hypoxic hearts compared with normoxic controls (Figure 7). To determine the effect of inhibition of PKC, p38 MAPK, JNK kinase, and p42/44 MAPK on resistance to myocardial ischemia, recovery of postischemic function was measured in normoxic and hypoxic hearts perfused with chelerythrine, SB203580, curcumin, and PD98059 before ischemia. Neither chelerythrine, SB203580, curcumin, nor PD98059 affected resistance to ischemia in normoxic hearts. In contrast, chelerythrine, SB203580, and curcumin completely abolished the cardioprotective effects of chronic hypoxia. PD98059 did not affect recovery of postischemic function in chronically hypoxic hearts. Recovery of postischemic function in the right ventricle for all drugs paralleled the change observed in the left ventricle.

## Discussion

Previously, we showed that chronic hypoxia in infant rabbits increases resistance of the heart to global ischemia.<sup>6</sup> However, the mechanisms by which hearts adapt to chronic hypoxia and resist subsequent ischemia remain unknown. In

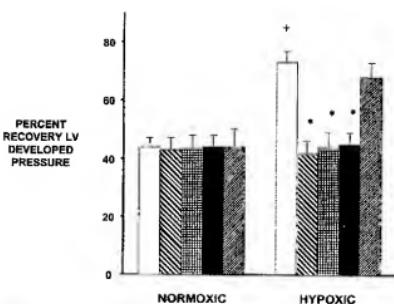


**Figure 6.** Parallel response of right atria and left ventricle to chronic hypoxia in infant rabbit. Chronic hypoxia resulted in activation of phospho-PKC $\epsilon$  and phospho-p38 MAPK in both left ventricle and right atria. Cyto indicates cytosolic; Part, particulate; LV, left ventricle; and RA, right atrium.

the present study, we have demonstrated that infant human and rabbit hearts adapt to chronic hypoxia through PKC $\epsilon$ , p38 MAPK, and JUN kinase activation but not p42/44 MAPK. Our data also reveal that many of the protein signaling mechanisms activated by chronic hypoxia in infant rabbits are identical to those activated in infant humans. Activation of PKC $\epsilon$ , p38 MAPK, and JUN kinase but not p42/44 MAPK mediates cardioprotection in chronically hypoxic infant rabbits.

#### Adaptation to Chronic Hypoxia

Chronically hypoxic human infant and rabbit hearts demonstrated activation of PKC $\epsilon$ , which was evident by translocation of the PKC $\epsilon$  isoform from the cytosolic to the particulate fraction. PKC $\epsilon$  but not the  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$ , and  $\zeta$  isoforms of PKC were phosphorylated and translocated in hearts adapted to chronic hypoxia. PKC $\epsilon$  is critical for cardiac myocyte protection by hypoxic preconditioning in a cell culture model.<sup>12</sup> Changes in specific PKC isoforms located in the myocardium have been reported, particularly in ischemic preconditioning, ischemia-reperfusion, heart failure caused by cardiomyopathy,



**Figure 7.** Recovery of left ventricular developed pressure in infant rabbit heart after 15 minutes of treatment with chelerythrine (1  $\mu$ mol/L), SB203580 (15  $\mu$ mol/L), curcumin (10  $\mu$ mol/L), and PD98059 (10  $\mu$ mol/L) before 30 minutes of global ischemia and 35 minutes of reperfusion. Control (□); chelerythrine (▨); SB203580 (▨); curcumin (▨); and PD98059 (▨). LV indicates left ventricle. Data are mean  $\pm$  SD ( $n=8$  hearts/group). + $P<0.05$ , normoxic vs hypoxic; \* $P<0.05$ , drugs vs control.

thy, and diabetes.<sup>7,13–15</sup> Our studies indicate activation of PKC $\epsilon$  is an important adaptive response to chronic hypoxia.

Chronic hypoxia results in activation of p38 MAPK and JUN kinase but not p42/44 MAPK in both human and rabbit hearts. Phosphorylation and activation of Hsp27 a substrate for p38 MAPK was present in chronically hypoxic infant hearts but not in normoxic hearts. We demonstrated that chronic hypoxia also caused phosphorylation of ATF-2, a substrate for p38 MAPK. We believe this is the first evidence of activation of protein kinase signaling pathways in infant human hearts in response to the stress of chronic hypoxia. In chronically hypoxic rabbit hearts, inhibition of PKC $\epsilon$  by chelerythrine prevents the activation and the translocation of PKC $\epsilon$  and p38 MAPK but not p42/44 MAPK. Inhibition of p38 MAPK by SB203580 did not inhibit PKC $\epsilon$  translocation in chronically hypoxic hearts. Thus in chronically hypoxic rabbit hearts, PKC $\epsilon$  appears upstream of the p38 MAPK pathway.

Adaptation to chronic hypoxia appears to stimulate phosphorylation of protein kinases to convert them from an inactive to an active state. Once activated, protein kinases translocate from the cytosolic to the particulate fraction, where their presence is associated with increased cardioprotection. Inhibition of activated PKC $\epsilon$ , p38 MAPK, and JNK reverses this chronic hypoxia-induced translocation of protein kinases, resulting in the abolition of cardioprotection. To explain this novel observation, we propose adaptation to hypoxia maintains protein kinases in a chronically active state with activation maintained by a mechanism involving continuous shuttling of protein kinases between the cytosolic and particulate fractions. These events would in turn maintain activation of nuclear transcription factors resulting in altered expression of target genes that confer cardioprotection.

#### Resistance to Myocardial Ischemia

Perfusion of rabbit hearts before ischemia with inhibitors of PKC $\epsilon$ , p38 MAPK, and JUN kinase alone abolished the cardioprotective

effects of chronic hypoxia but had no effect in normoxic hearts. Inhibition of p42/44 MAPK by PD98059 before ischemia had no effect on cardioprotection in normoxic and chronically hypoxic hearts, confirming our findings that p42/44 MAPK does not play a role in chronically hypoxic hearts.

Cardioprotection induced by adaptation to chronic hypoxia may involve changes in the actin cytoskeleton. Activation of p38 MAPK activates MAPKAP-2, which can in turn phosphorylate Hsp27,<sup>16</sup> an important regulator of actin dynamics that promotes polymerization of actin filaments, thus increasing the stability of the cytoskeleton.<sup>17</sup> Activation of p38 MAPK has been shown to prevent cytochalasin D-induced fragmentation of actin filaments, thus preserving cell viability.<sup>17,18</sup> Furthermore, overexpression of Hsp27 in isolated rat ventricular myocytes confers protection against simulated ischemia.<sup>11</sup> Because prolonged ischemia is known to cause cytoskeleton disruption, activation of the MAPKAPK-2/Hsp27 pathway and preservation of the actin filaments may explain some of the cardioprotective effects of adaptation to chronic hypoxia. In addition, phosphorylated Hsp27 interacts with Daxx, a mediator of Fas-induced apoptosis, preventing the interaction of Daxx with both Ask1 and Fas to block Daxx-mediated apoptosis.<sup>19</sup> Cardioprotection by adaptation to chronic hypoxia is also associated with activation of sarcolemmal and mitochondrial K<sub>ATP</sub> channels.<sup>20</sup> PKC activates the sarcolemmal K<sub>ATP</sub> channel by phosphorylation of the pore forming Kir6.2 subunit.<sup>21</sup> Thus, activation of PKC by chronic hypoxia may mediate cardioprotection by regulating K<sub>ATP</sub> channel function.

The limitations of our study are that we could not identify the cell type in which PKCε and MAPKs are activated. In addition, resistance to ischemia in hearts from human infants at the time of surgical repair was not measured. The proposed role of PKC and MAPKs in the signal transduction pathway by which infant hearts adapt to chronic hypoxia and resist subsequent ischemia has been based on experiments with kinase inhibitors. This pharmacological approach is dependent on the relative specificity of the inhibitors. For example, SB203580 inhibits p38α, β, and β2 but not γ and δ isoforms of p38 MAPK. SB203580 does not inhibit PKC and JNK. Chelerythrine inhibits all PKC isoforms and can activate MAPK pathways. Curcumin inhibits several kinases upstream of JNK and is an antioxidant. PD98059 is a potent and selective inhibitor of MEK, an upstream kinase of p42/44 MAPK.

We conclude infant human and rabbit hearts adapt to chronic hypoxia through activation of PKCε, p38 MAPK, and JUN kinase. It appears that these pathways are responsible for cardioprotection in the chronically hypoxic infant rabbit heart. Protection of the infant heart during surgical repair of congenital heart defects remains incomplete.<sup>22</sup> Exploitation of one or more of these protein kinase signaling pathways may afford increased cardioprotection to human infants undergoing repair of congenital heart defects.

### Acknowledgments

This work was supported by grants HL-54075 (Dr Baker), HL-61414 (Dr Pritchard) from the National Heart, Lung, and Blood Institute,

Ronald McDonald's Children's Charities (Dr Baker), and Children's Hospital of Wisconsin Foundation (Drs Tweddell and Baker).

### References

- Goldberg M, Zhang HL, Steinberg SF. Hypoxia alters the subcellular distribution of protein kinase C isoforms in neonatal rat ventricular myocytes. *J Clin Invest*. 1997;99:55-61.
- Sugden PH, Clerk A. "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circ Res*. 1998;83:345-352.
- Cook SA, Sugden PH, Clerk A. Activation of c-Jun N-terminal kinases and p38 mitogen-activated protein kinases in human heart failure secondary to ischemic heart disease. *J Mol Cell Cardiol*. 1999;31:1429-1434.
- Talmor D, Applebaum A, Rudich A, et al. Activation of mitogen-activated protein kinases in human heart during cardiopulmonary bypass. *Circ Res*. 2000;86:1004-1007.
- Shi Y, Pritchard JK Jr, Holman P, et al. Chronic myocardial hypoxia increases nitric oxide synthase and decreases caveolin-3. *Free Radic Biol Med*. 2000;29:695-703.
- Baker JE, Holman P, Gross GE. Preconditioning in immature rabbit hearts: role of KATP channels. *Circulation*. 1999;99:1249-1254.
- Ping P, Zhang J, Qiu Y, et al. Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res*. 1997;81:404-414.
- Seko Y, Takahashi N, Tobe K, et al. Hypoxia and hypoxia/reoxygenation activate p65PAK, p38 mitogen-activated protein kinase (MAPK), and stress-activated protein kinase (SAPK) in cultured rat cardiac myocytes. *Biochem Biophys Res Commun*. 1997;239:840-844.
- Fryer R, Pratt P, Hsu A, et al. Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. *J Pharmacol Exp Ther*. 2001;296:642-649.
- Maulik N, Watanabe M, Zu Y, et al. Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. *FEBS Lett*. 1996;396:233-237.
- Li L, Li JL, Mesari R, et al. Small heat shock proteins and protection against ischemic injury in cardiac myocytes. *Circulation*. 1997;96:4343-4348.
- Gray MO, Karliner JS, Mochly-Rosen D. A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem*. 1997;272:30945-30951.
- Bowling N, Walsh RA, Song G, et al. Increased protein kinase C activity and expression of Ca<sup>2+</sup>-sensitive isoforms in the failing human heart. *Circulation*. 1999;99:384-391.
- Strasser RH, Simonis G, Schon SP, et al. Two distinct mechanisms mediate a differential regulation of protein kinase C isozymes in acute and prolonged myocardial ischemia. *Circ Res*. 1999;85:77-87.
- Inoguchi T, Battan R, Handler E, et al. Preferential elevation of protein kinase C isozyme beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A*. 1992;89:11059-11063.
- Freshney NW, Ravineen L, Guesdon F, et al. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell*. 1994;78:1039-1049.
- Guy J, Lambert H, Gingras-Breton G, et al. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. *J Cell Sci*. 1997;110:357-368.
- Huot J, Houle F, Spitz DR, et al. HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. *Cancer Res*. 1996;56:273-279.
- Charette SJ, Laviole JN, Lambert H, et al. Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol Cell Biol*. 2000;20:7602-7612.
- Kong X, Tweddell J, Gross G, et al. Sarcolemmal and mitochondrial K<sub>ATP</sub> channels mediate cardioprotection in chronically hypoxic hearts. *J Mol Cell Cardiol*. 2001;33:1041-1046.
- Light PE, Bladen C, Windle RJ, et al. Molecular basis of protein kinase C-induced activation of ATP-sensitive potassium channels. *Proc Natl Acad Sci U S A*. 2000;97:9058-9063.
- Imura H, Caputo M, Parry A, et al. Age-dependent and hypoxia-related differences in myocardial protection during pediatric open heart surgery. *Circulation*. 2001;103:1551-1556.